

## DESCRIPTION

Tumor Antigen Proteins, Genes Therefor, and Tumor Antigen Peptides

## TECHNICAL FIELD

The present invention relates to medicines for activating  
5 antitumor immunity and for treating autoimmune diseases as well as to  
diagnosis of tumors or autoimmune diseases. In particular, the present  
invention relates to novel tumor antigen proteins, novel genes therefor,  
novel tumor antigen peptides, and the like.

## PRIOR ART

10 It is known that the immune system, particularly T cells, plays  
an important role *in vivo* in tumor rejection. Indeed, infiltration of  
lymphocytes having cytotoxic effects on tumor cells has been observed  
in human tumor foci (*Arch. Surg.*, 126:200-205, 1990), and cytotoxic T  
lymphocytes (CTLs) recognizing autologous tumor cells have been  
15 isolated from melanomas without great difficulties (e.g., *Immunol.*  
*Today*, 8:385, 1987; *J. Immunol.*, 138:989, 1987; and *Int. J. Cancer*,  
52:52-59, 1992). In addition, the results of clinical treatment of  
melanomas by T cell introduction also suggest the importance of T cells  
in tumor rejection (*J. Natl. Cancer. Inst.*, 86:1159, 1994).

20 Although it has long been unknown about target molecules for  
CTLs attacking autologous tumor cells, the recent advance in  
immunology and molecular biology has gradually begun elucidating such  
target molecules. Specifically, it has been found that using T cell  
receptors (TCRs), CTL recognizes a complex consisting of tumor  
25 antigen peptide and major histocompatibility complex (MHC) class I

antigen, and thereby attacks autologous tumor cells.

Tumor antigen peptides are generated from tumor antigen proteins. Thus, the proteins are intracellularly synthesized and then degraded in cytoplasm into the peptides by proteasome. On the other hand, MHC class I antigens formed at endoplasmic reticulum bind to the above tumor antigen peptides, and are transported via cis Golgi to trans Golgi, *i.e.*, the mature side, and expressed on the cell surface (*Rinsho-Menneki*, 27(9):1034-1042, 1995).

As such a tumor antigen protein, T. Boon *et al.* identified a protein named MAGE from human melanoma cells for the first time in 1991 (*Science*, 254:1643-1647, 1991), and thereafter several additional tumor antigen proteins have been identified from melanoma cells.

As described in the review by T. Boon *et al.* (*J. Exp. Med.*, 183, 725-729, 1996), tumor antigen proteins hitherto identified can be divided into the following four categories.

Tumor antigen proteins belonging to the first category are those proteins which are expressed only in testis among normal tissues, while they are expressed in melanoma, head and neck cancer, non-small cell lung cancer, bladder cancer and others, among tumor tissues. Among tumor antigen proteins in this category are the above-described MAGE and analogous proteins constituting a family of more than 12 members (*J. Exp. Med.*, 178:489-495, 1993), as well as BAGE (*Immunity*, 2:167-175, 1995) and GAGE (*J. Exp. Med.*, 182:689-698, 1995), all of which have been identified from melanoma cells.

Although some of such tumor antigen proteins in this category

are highly expressed in melanoma, their expression is observed in only 10 to 30% of patients having a particular tumor other than melanoma, and therefore, they can not be applied widely to treatments or diagnoses of various tumors.

5 Tumor antigen proteins belonging to the second category are those proteins which are expressed only in melanocytes and retina among normal tissues, while the expression is observed only in melanomas among tumor tissues. Since these tissue-specific proteins are highly expressed in melanomas, they function as tumor antigen proteins specific  
10 for melanomas. Among tumor antigen proteins in this category are tyrosinase (*J. Exp. Med.*, 178:489-495, 1993), MART-1 (*Proc. Natl. Acad. Sci. USA*, 91:3515, 1994), gp100 (*J. Exp. Med.*, 179:1005-1009, 1994), and gp75 (*J. Exp. Med.*, 181:799-804, 1995), genes for which have all been cloned from melanoma cells. Additionally and separately  
15 identified Melan-A (*J. Exp. Med.*, 180:35, 1994) has proved to be the same molecule as MART-1.

However, the tumor antigen proteins in this category can not be used widely for treatments or diagnoses of various tumors, since they are not expressed in tumors other than melanoma.

20 Tumor antigen proteins belonging to the third category are those proteins which yield, through tumor-specific mutations, tumor antigen peptides newly recognized by CTL. Among tumor antigen proteins in this category are mutated CDK4 (*Science*, 269:1281-1284, 1995),  $\beta$ -catenin (*J. Exp. Med.*, 183:1185-1192, 1996), and MUM-1  
25 (*Proc. Natl. Acad. Sci. USA*, 92:7976-7980, 1995). In CDK4 and  $\beta$ -

catenin, a single amino acid mutation increases the binding affinity of the peptides to MHC class I antigen, and allows them to be recognized by T cells. In MUM-1, its intron normally untranslated is translated due to mutation, and the peptide thus generated is recognized by T cells.

5 However, since such mutations occur at low frequency, they can not be applied widely to treatments or diagnoses of various tumors.

As a tumor antigen protein belonging to the fourth category, P15 has been identified from melanoma cells, which is a protein widely expressed in normal tissues and which is also recognized by CTL (*J.*

10 *Immunol.* 154:5944-5955, 1995).

Tumor antigen proteins or peptides hitherto known have been identified as follows.

In such identification, a set of tumor cells and CTLs attacking the tumor cells (usually established from lymphocytes of the same patient from whom the tumor cells are obtained) are firstly provided. Then, the cells from this set are used to directly identify tumor antigen peptides, or used to determine the gene encoding the tumor antigen protein from which corresponding tumor antigen peptides are identified.

Specifically, in the case where tumor antigen peptides are directly identified, tumor antigen peptides bound to MHC class I antigens in tumor cells are extracted under acidic conditions, and separated into various peptides using high-performance liquid chromatography. Cells expressing MHC class I antigen, but not expressing tumor antigen protein (for example, B cells from the same patient), are then pulsed with such various peptides, and examined for

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their reactivity with CTL to identify tumor antigen peptides. Then, the sequences of the peptides thus identified are further determined by, for example, mass spectrometry. In this way, tumor antigen peptides derived from Pmel 17 which is the same molecule as gp100 have been identified from melanoma cells (*Science*, 264:716-719, 1994).

In order to firstly determine the gene encoding tumor antigen protein and then to identify therefrom corresponding tumor antigen peptides, the gene encoding tumor antigen protein may be cloned using molecular biological techniques. cDNAs are prepared from tumor cells, and cotransfected with MHC class I antigen gene into cells not expressing tumor antigen proteins (for example, COS cells), in order to express them transiently. The products thus expressed are then repeatedly screened on the basis of their reactivity with CTL, until the gene encoding tumor antigen protein may finally be isolated. In this way, the genes for the above-mentioned MAGE, tyrosinase, MART-1, gp100, and gp75 have been cloned.

In order to deduce and identify the presented tumor antigen peptides actually bound to MHC class I antigens on the basis of the information about such tumor antigen gene, the methods as described below are used. Firstly, fragments of the gene encoding tumor antigen protein, having various sizes, are prepared using, for example, PCR, exonucleases, or restriction enzymes, and cotransfected with MHC class I antigen gene into cells not expressing tumor antigen proteins (e.g., COS cells), in order to express them transiently. The region(s) which include tumor antigen peptides are then identified on the basis of their

reactivity with CTL. Subsequently, peptides are synthesized. Cells expressing MHC class I antigen but not expressing tumor antigen proteins are then pulsed with the synthesized peptides, and examined for their reactions with CTL to identify the tumor antigen peptides (*J. Exp.*

5 *Med.*, 176:1453, 1992; *J. Exp. Med.*, 179:24, 759, 1994). The sequence regularities (motifs) for peptides, which are bound and presented by certain types of MHC such as HLA-A1, -A0201, -A0205, -A11, -A31, -A6801, -B7, -B8, -B2705, -B37, -Cw0401, and -Cw0602 have been known (*Immunogenetics*, 41:178-228, 1995), and therefore, candidates for tumor antigen peptides may also be designed by making  
10 reference to such motifs, and such candidate peptides may be practically synthesized and examined in the same way as described above (*Eur. J. Immunol.*, 24:759, 1994; *J. Exp. Med.*, 180:347, 1994).

Furthermore, it is another possibility that tumor antigen  
15 proteins expressed at high level in tumors are expressed also in normal tissues, and cause autoimmune diseases by inducing excessive immune response against such tumor antigen proteins. For example, it was reported that when a combination of a chemotherapeutic agent and IL-2 was used for treating melanomas, appearance of leukoderma was  
20 observed (*J. Clin. Oncol.*, 10:1338-1343, 1992). This is probably because CTLs or antibodies against the complexes consisting of fragments of the tumor antigen protein expressed in melanomas (referred to as peptide fragments) and MHC class I antigens were inductively produced, and they affected normal skin tissues to develop leukoderma,  
25 an autoimmune disease-like symptom.

**SUBJECT THAT THE INVENTION IS TO SOLVE**

As described above, some of the known tumor antigen proteins are expressed only in limited tumors, and others are expressed only in a small number of patients having a particular tumor even if they are  
5 expressed in various kinds of tumor, and therefore, they can not be used widely for treatments or diagnoses of various tumors.

Thus, the present invention aims to provide tumor antigen proteins or fragments thereof (hereinafter referred to as "peptide fragments" or as "tumor antigen peptides") which, unlike the known  
10 tumor antigen proteins or their peptide fragments, can be used for treatments or diagnoses of a wide variety of tumors including squamous cell carcinoma, or which can be applied to major part of patients having a particular tumor even if they can be used only for limited tumors, or which can be applied to various tumors as a therapeutic or diagnostic  
15 assistant in the treatment or diagnosis for such tumors.

Squamous cell carcinoma is one of the most common cancers in human. In particular, squamous cell carcinomas in esophageal cancer and lung cancer are known to be relatively resistant to current chemotherapy and radiotherapy. Also in this regard, it is desired to  
20 develop specific immunotherapies such as those which use tumor antigen proteins or corresponding tumor antigen peptides.

Furthermore, when one develops autoimmune disease due to excessively induced specific immunity raised by tumor antigen protein, it would be desirous treatments to specifically block such immune response  
25 using, for example, antisense DNA/RNA for the gene encoding tumor

antigen proteins or antagonists for the tumor antigen peptides.

# MEANS FOR SOLVING THE SUBJECT

With the aim of obtaining tumor antigen protein or corresponding tumor antigen peptides which can be applied widely to treatments or diagnoses of various tumors including, in particular, squamous cell carcinoma, the present inventors tried to identify tumor antigen proteins from tumors other than melanoma.

Specifically, the present inventors established a squamous cell carcinoma cell line KE-4 derived from esophageal cancer (hereinafter referred to as esophageal cancer cell line KE-4 or simply as KE-4), and also established CTL (hereinafter referred to as KE-4CTL) which recognizes tumor antigen peptides restricted to HLA-A2601 which is a MHC class I antigen expressed in said KE-4 (*Cancer Res.*, 55:4248-4253, 1995).

Fibroblast cell line VA-13 was then cotransfected with a recombinant plasmid of cDNA library prepared from KE-4 and a recombinant plasmid containing HLA-A2601 cDNA. The resulting transfectants were treated with KE-4CTL, and screened by measuring the amount of produced IFN- $\gamma$  to determine whether KE-4-CTL was activated. As a result, the inventors succeeded in cloning a novel gene encoding tumor antigen protein of the present invention for the first time from tumor cells other than melanoma.

Thus, the gist of the present invention relates to :

(1) DNA encoding a protein having the amino acid sequence shown in

SEQ ID NO: 1 or a variant protein thereof in which one or more amino

2 (Identification Method: P)



acid residues are substituted, deleted or added, said protein and variant protein thereof being capable of yielding, through its intracellular decomposition, peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state;

5 (2) DNA which comprises the base sequence shown in SEQ ID NO: <sup>1 (Identification methods)</sup> 2, or a variant DNA which hybridizes to said DNA under stringent conditions, the protein produced by expression of said DNA and variant DNA being capable of yielding, through its intracellular decomposition, peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state;

(3) medicines comprising DNA of the above item (1) or (2) as an active ingredient;

(4) expression plasmids comprising DNA of the above item (1) or (2);

15 (5) transformants transformed with the expression plasmid of the above item (4);

(6) tumor antigen proteins produced by expression of DNA of the above item (1) or (2);

20 (7) tumor antigen peptides comprising part of the protein of the above item (6) which can bind to MHC class I antigen to be recognized by T cells, or derivatives thereof having functionally equivalent properties;

25 (8) tumor antigen peptides of the above item (7) which comprise all or part of the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 in the amino acid sequence of SEQ ID NO: <sup>2</sup> 2, or derivatives thereof having functionally equivalent properties;

(9) medicines comprising, as an active ingredient, tumor antigen protein

of the above item (6), tumor antigen peptide or derivative thereof defined in the above item (7) or (8).

(10) antibodies which specifically bind to the tumor antigen proteins of the above item (6) or tumor antigen peptides of the above item (7) or

5 (8); and

(11) DNA comprising 8 or more bases having a sequence complementary to the coding or 5' non-coding sequence of DNA having the base

*B* sequence shown in SEQ ID NO: <sup>2</sup>~~2~~, or RNA corresponding to said DNA, or chemically modified variant thereof.

## 10 MODE FOR CARRYING OUT THE INVENTION

DNAs of the present invention encode a novel tumor antigen protein, and may include a DNA which encodes a protein having the amino acid sequence shown in SEQ ID NO: <sup>2</sup>~~1~~ or a variant protein thereof in which one or more amino acid residues are substituted, deleted or

*B* 15 added, said protein and variant protein being capable of yielding, through its intracellular decomposition, peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in

such binding state, as well as DNA which comprises the base sequence shown in SEQ ID NO: <sup>1</sup>~~2~~ or variant DNA thereof which hybridizes to said

20 DNA under stringent conditions, the protein produced by expression of said DNA and variant DNA being capable of yielding, through its intracellular decomposition, peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state.

25 As used herein, the phrase "variant protein thereof in which

one or more amino acid residues are substituted, deleted, or added" refers to so-called variant proteins artificially prepared, to naturally-occurring polymorphism, or to proteins produced by mutation or modification but having functionally equivalent properties. DNAs encoding such variant proteins may be prepared using, for example, the methods described in Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., vols. 1-3 (Cold Spring Harbor Laboratory Press, New York, 1989), such as site-directed mutagenesis or PCR method. In this context, the number of amino acid residues to be substituted, deleted, or added should be such a number that permits the substitution, deletion or addition by well-known methods such as site-directed mutagenesis described above.

"Variant DNA which hybridizes to DNA under stringent conditions" as described herein may be obtained using, for example, the methods described in *Molecular Cloning* mentioned above. In this context, "stringent conditions" refers to, for example, such conditions that hybridization is conducted at 42°C in a solution containing 6x SSC (20x SSC means 333 mM sodium citrate and 333 mM NaCl), 0.5% SDS, and 50% formamide, followed by washing in a solution of 0.1x SSC and 0.5% SDS at 68°C, or those conditions described in Nakayama *et al.*, *Bio-Jikken-Illustrated*, vol. 2, "Idenshi-Kaiseki-no-Kiso (Basis for Gene Analysis)", pp. 148-151, Shujunsha, 1995. For the purpose of this invention, the protein produced by expression of such hybridizable DNA should comprise a peptide segment which is capable of binding to MHC class I antigen and recognized by T cells.

As used herein, "protein and variant protein which are capable of yielding, through its intracellular decomposition, peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state" (hereinafter, such protein is sometimes referred to as tumor antigen protein) means that partial peptide consisting of part of the amino acid sequence of such protein or variant protein can bind to MHC class I antigen, and that when bound to MHC class I antigen and presented on cell surface, the complex of the peptide fragment and MHC class I antigen can be recognized by T cells capable of specifically binding thereto, and transduces signals to T cells. In this context, such binding means non-covalent binding.

In order to confirm that a given peptide fragment is capable of binding to MHC class I antigen and recognized by T cells, the peptide fragment may be bound to MHC class I antigen and presented on cell surface by expressing it endogenously in an appropriate cell or by adding it exogenously to an appropriate cell (pulsing). The peptide presenting cells may be then treated with T cells specific to the tumor antigen protein, and cytokines produced by the T cells may be measured. Alternatively, as a method measuring the cytotoxic activity of T cells against the peptide-presenting cells, a method using the peptide-presenting cells labeled with  $^{51}\text{Cr}$  (*Int. J. Cancer*, 58:317 (1994)) may also be used. In such methods, CTLs are preferably used as the T cells recognizing the peptide.

DNA of the present invention may be used as an active ingredient of medicines. For example, medicines which comprise DNA

of the present invention as an active ingredient can be used for treating or preventing tumors by administering the DNA of the present invention to tumor patients. When DNA of the present invention is administered, the tumor antigen protein is expressed at high level in the cells. As a result, the tumor antigen peptides bind to MHC class I antigen and presented on the cell surface at high density. This will cause efficient proliferation of tumor-specific CTLs in the body, allowing treatment or prevention of the tumor. Administration and introduction of DNA of the present invention into cells may be achieved using viral vectors or according to any one of other procedures (*Nikkei-Science*, April, 1994, pp. 20-45; *Gekkan-Yakuji*, 36(1), 23-48 (1994); *Jikken-Igaku-Zokan*, 12(15), 1994, and references cited therein).

Examples of the methods using viral vectors include those methods in which DNA of the present invention is incorporated into DNA or RNA virus such as retrovirus, adenovirus, adeno-associated virus, herpesvirus, vaccinia virus, poxvirus, poliovirus, or Sindbis virus, and introduced into cells. Among them, the methods using retrovirus, adenovirus, adeno-associated virus, or vaccinia virus are particularly preferred.

Other methods may include those in which expression plasmids are directly injected intramuscularly (DNA vaccination), the liposome method, Lipofectin method, microinjection, the calcium phosphate method, and electroporation, with DNA vaccination and the liposome method being particularly preferred.

In order to make DNA of the present invention act as medicine

in practice, one can use either of two methods: *in vivo* method in which DNA is directly introduced into the body, or *ex vivo* method in which certain cells are removed from human, and after introducing DNA into said cells extracorporeally, reintroduced into the body (*Nikkei-Science*, 5 April, 1994, pp. 20-45; *Gekkan-Yakuji*, 36(1), 23-48 (1994); *Jikkenn-Igaku-Zokan*, 12(15), 1994; and references cited therein). *In vivo* method is rather preferred.

In the case of *in vivo* methods, DNA may be administered by any appropriate route depending on the diseases and symptoms to be 10 treated, and other factors. For example, it may be administered by intravenous, intraarterial, subcutaneous, intracutaneous, or intramuscular routes. In the case of *in vivo* methods, such medicines may be administered in various dosage forms such as solution, and they are typically formulated into injections containing DNA of the present 15 invention as an active ingredient, which may also include, if necessary, conventional carriers. When DNA of the present invention is included in liposomes or membrane-fused liposomes (such as Sendai virus (HVJ)-liposomes), such medicines may be in the form of suspension, frozen drug, centrifugally-concentrated frozen drug or the like.

20 Although the amount of DNA of the present invention in such formulations may vary depending on, for example, the disease to be treated, the age and body weight of a particular patient, it is usually preferred to administer 0.0001-100 mg, more preferably 0.001-10 mg, of DNA of the present invention every several days to every several 25 months.

Furthermore, the tumor antigen protein can be prepared in large quantities by recombinant DNA techniques using DNA of the present invention.

Preparation of tumor antigen protein by expression of DNA of the present invention may be achieved according to many publications and references such as *Molecular Cloning* mentioned above. An expression plasmid which can replicate and function in host cells is constructed by adding regulatory gene(s) such as a promoter which controls transcription (e.g., trp, lac, T7, or SV40 early promoter) upstream to the DNA to be expressed and by inserting the resultant DNA into an appropriate vector (e.g., pSV-SPORT1). The expression plasmid is then introduced into appropriate host cells to obtain transformants. Examples of host cell include, for example, prokaryotes such as *Escherichia coli*, unicellular eukaryotes such as yeast, and cells derived from multicellular eukaryotes such as insects or animals. Gene transfer into host cells may be achieved by, for example, the calcium phosphate method, DEAE-dextran method, or the electric pulse method. Transformants cultured in appropriate medium produce the protein of interest. The tumor antigen protein thus obtained may be isolated and purified according to standard biochemical procedures.

In the present invention, "peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state", which may be produced through intracellular decomposition of tumor antigen protein of the present invention, *i.e.*, "tumor antigen peptides", may be determined as follows.

Firstly, fragments of DNA encoding tumor antigen protein and having various sizes are prepared using, for example, PCR, exonucleases, or restriction enzymes, and then inserted into expression vectors as described above. The vectors are then cotransfected into cells not  
5 expressing tumor antigen proteins (e.g., COS cells), with a plasmid which comprises a gene for MHC class I antigen that presents tumor antigens, in order to express them transiently. The regions which include the tumor antigen peptides are identified on the basis of the reactivity of the transfectants with CTL. Subsequently, various  
10 peptides included in such regions are synthesized. Cells expressing MHC class I antigen which presents tumor antigens but not expressing tumor antigen proteins are pulsed with the synthesized peptides, and examined for their reaction with CTL to identify the tumor antigen peptides (*J. Exp. Med.*, 176:1453, 1992; *J. Exp. Med.*, 179:24, 759,  
15 1994).

Alternatively, the sequence regularities (motifs) of antigen peptides bound and presented by certain MHC types such as HLA-A1, -A0201, -A0205, -A11, -A24, -A31, -A6801, -B7, -B8, -B2705, -B37, -Cw0401, and -Cw0602 have been known, and therefore, candidates for  
20 tumor antigen peptides may also be selected making reference to such motifs, and such candidate peptides may be synthesized and identified in the manner as described above (*Eur. J. Immunol.*, 24:759, 1994; *J. Exp. Med.*, 180:347, 1994).

It is also known that MHC includes class II antigens besides  
25 class I antigens, and that conjugates of such MHC class II antigen with



particular tumor antigen peptides, which may be produced from tumor antigen protein through phagocytosis and decomposition by antigen-presenting cells, such as macrophage, will activate tumor-specific helper T cells (*J. Immunol.*, **146**:1708-1714, 1991).

5           The successful cloning of the novel tumor antigen protein gene of the present invention also enables those skilled in the art to determine additional tumor antigen peptides which bind to MHC class II antigen described above. Specifically, such antigen peptides may be determined on the basis of their reactivity with T cells or based on  
10 known information on motifs of such antigen peptides, in the same manner as MHC class I antigen.

          The tumor antigen peptides thus determined may be prepared by usual methods known in peptide chemistry such as those described in "*Peptide Synthesis*" (Interscience, New York, 1966), "*The Proteins*"  
15 (vol. 2, Academic Press Inc., New York, 1976), "*Pepuchido-Gosei*" (Maruzen, 1975), or "*Pepuchido-Gosei-no-Kiso-to-Jikkenn*" (Maruzen, 1985). In particular, such peptide can be synthesized by selecting either the liquid phase method or the solid phase method depending on the structure of its C-terminus, with the liquid phase method being more  
20 preferable. Thus, peptides may be prepared by protecting and deprotecting functional groups in amino acids, and elongating them by a single residue or several residues. Protecting groups for functional groups on amino acids are described, for example, in the above-mentioned publications concerning peptide chemistry.

25           For the purpose of the present invention, "tumor antigen

peptides" may be defined as peptide fragments derived from either a protein having the amino acid sequence shown in SEQ ID NO: <sup>2</sup>~~1~~ or a variant protein thereof as defined above. Although the following description mainly relates to tumor antigen peptides derived from the protein having the amino acid sequence shown in SEQ ID NO: <sup>2</sup>~~1~~ as well as derivatives thereof, it will be understood that such description can apply to tumor antigen peptides derived from variant proteins.

Tumor antigen peptides produced by intracellular decomposition of the protein shown in SEQ ID NO: <sup>2</sup>~~1~~ are not specifically restricted, and may include, but not limited to, those peptides that comprise all or part of the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 in the amino acid sequence shown in SEQ ID NO: <sup>2</sup>~~1~~. Preferred are those peptides that consist of 9 amino acid residues, and those peptides that consists of the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 in SEQ ID NO: <sup>2</sup>~~1~~ are particularly preferred. Regarding tumor antigen peptides described herein, for example, the peptide consisting of the amino acid sequence of positions 749-757 in SEQ ID NO: <sup>2</sup>~~1~~ is sometimes abbreviated as "749-757".

As used herein, "derivatives of tumor antigen peptide" refers to those derivatives which have properties functionally equivalent to such tumor antigen peptide and in which some of the amino acid residues in said peptide are substituted, deleted, or added, or to those derivatives in which amino group(s) or carboxy group(s) in said peptide(s) or derivatives described just above are modified. In particular, examples

of such derivatives may include those derivatives in which, in a tumor antigen peptide of the present invention comprising all or part of the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 in the amino acid sequence of SEQ ID NO: <sup>2</sup>~~1~~, some of the amino acid residues in the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 are substituted or deleted, or other amino acid residue(s) are added thereto.

Among derivatives in which some of the amino acid residues in said peptide are substituted, deleted, or added, preferred are those derivatives which retain the epitope regions in the tumor antigen peptides involved in their binding with CTL and in which amino acid residue(s) in the tumor antigen peptides involved in their binding with MHC class I antigen are substituted, deleted, or added. Among such derivatives, those derivatives in which a single amino acid residue is substituted are more preferred (*Immunol.* 84:298-303, 1995). For antigen peptides derived from melanoma tumor antigen protein gp 100, it is reported that substitution of amino acid(s) in the binding site for MHC class I antigen has resulted in its stronger binding with MHC class I antigen, and also caused stronger induction of CTL specific to such antigen peptide when used in *in vitro* stimulation of peripheral blood lymphocytes derived from melanoma patients (*J. Immunol.*, 157:2539-2548, 1996).

Such derivatives can be easily synthesized using a commercially available peptide synthesizer, and the binding affinity of synthesized derivatives to MHC class I antigen may be easily measured

by competitive inhibition assay between said derivatives and radiolabeled standard peptide for binding to MHC class I antigen (R. T. Kubo *et al.*, *J. Immunol.*, 152:3913, 1994). Thus, by subjecting various peptide derivatives to such assay, peptide derivatives having CTL-inducing activity can be easily selected. Since the peptide derivatives thus selected can bind to MHC class I antigen more strongly while retaining their binding ability to CTL, they can be used as more efficient tumor antigen peptides.

Examples of modifying group for amino group may include acyl groups, and in particular, alkanoyl groups of 1-6 carbon atoms, alkanoyl groups of 1-6 carbon atoms substituted by phenyl group, carbonyl groups substituted by cycloalkyl group of 5-7 carbon atoms, alkylsulfonyl groups of 1-6 carbon atoms, phenylsulfonyl groups, and the like.

Modifying group for carboxy group include, for example, ester and amide groups. Specific examples of such ester group may be alkyl ester groups of 1-6 carbon atoms, alkyl ester groups of 0-6 carbon atoms substituted by phenyl group, and cycloalkyl ester groups of 5-7 carbon atoms, and specific examples of such amide group may be an amide group, amide groups substituted by one or two alkyl groups of 1-6 carbon atoms, amide groups of 0-6 carbon atoms substituted by one or two alkyl groups substituted by phenyl, and amide groups forming a 5-7 membered azacycloalkane including the amide nitrogen as a ring member.

"Antibodies" of the present invention may be easily prepared according to, for example, the methods described in Lane, H.D. *et al.*,

*Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York, 1989). Specifically, antibodies which recognize tumor antigen proteins or tumor antigen peptides, and antibodies which further neutralize their activities may be easily prepared by immunizing an animal with such tumor antigen protein or tumor antigen peptide using conventional procedures. Such antibodies may be used in, for example, affinity chromatography, screening of cDNA library, immunological diagnosis, or preparation of medicines. Such immunological diagnosis may include immunoblotting, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), fluorescent or luminescent assay, and the like.

As used herein, "DNA comprising 8 or more bases having a sequence complementary to the coding sequence or 5' non-coding sequence of DNA comprising the base sequence shown in SEQ ID NO. <sup>1</sup>~~2~~, or RNA corresponding to said DNA" means an antisense strand of double stranded DNA, or RNA corresponding to such antisense strand DNA, comprising 8 or more bases (hereinafter referred to as antisense oligonucleotides).

For example, as such antisense oligonucleotides, DNA may be prepared on the basis of the base sequence of the gene encoding tumor antigen protein of the present invention, and corresponding RNA may be prepared by incorporating such DNA into an expression plasmid in the antisense direction.

Although such antisense oligonucleotides may have a sequence complimentary to any part of the coding sequence or 5' non-coding

sequence of DNA of the present invention comprising the base sequence shown in SEQ ID NO: <sup>1</sup>2, they preferably have a sequence complimentary to transcription initiation site, translation initiation site, 5' non-translated region, a boundary region between exon and intron, or 5' CAP region.

In the above description, "chemically modified variants" of "DNA or RNA corresponding to said DNA" (hereinafter referred to as chemically modified variant of antisense oligonucleotides) may include those variants which have increased transferability into cells or increased stability in cells. Specific examples of the variants include phosphorothioate, phosphorodithioate, alkyl phosphotriester, alkyl phosphonate, or alkyl phosphoamidate derivatives ("*Antisense RNA and DNA*", WILLEY-LISS, 1992, pp. 1-50). Such chemically modified variant may be prepared according to, for example, the above-mentioned reference.

Such antisense oligonucleotides or chemically modified variants thereof may be used to control expression of the gene encoding tumor antigen protein. Since such control can decrease the amount of tumor antigen protein to be produced, and thereby decrease a damage caused by CTLs and also inhibit proliferation of CTL, autoimmune diseases due to over-expression of tumor antigen protein may be treated or prevented by such approach.

When the antisense oligonucleotides or chemically modified variants thereof are administered as such, preferred length thereof may be 8-200 bases, more preferably 10-25 bases, and most preferably 12-25

bases.

When inserted into expression plasmids, preferred length of the antisense oligonucleotides may be 100 bases or more, preferably 300-1000 bases, and more preferably 500-1000 bases.

5           Antisense oligonucleotides inserted in expression plasmids may be introduced into cells according to, for example, the methods described in *Jikken-Igaku*, vol. 12 (1994), such as those employing liposomes or recombinant viruses. Expression plasmids for antisense oligonucleotides may be easily prepared using conventional expression  
10           vectors just by placing the genes of the present invention after the promoter in the opposite direction so that the genes of the present invention may be transcribed in the direction from 3' to 5'.

When administered as such, antisense oligonucleotides or chemical variants of the antisense oligonucleotides may be formulated by  
15           mixing them with stabilizing agents, buffers, solvents, and/or the like, and then administered simultaneously with antibiotics, anti-inflammatory agents, or anesthetics. The formulations thus prepared may be administered via various routes. Such formulations are preferably administered everyday or every several days to every several weeks.

20           Furthermore, in order to avoid such frequent administration, sustained-release minipellet formulation may also be prepared and implanted near the affected area. Alternatively, the formulation may be slowly administered in continuous manner using, for example, an osmotic pump. Dosage are typically to be adjusted so that the concentration at the site  
25           of action will be from 0.1 nM to 10  $\mu$ M.

Tumor antigen proteins, tumor antigen peptides, and derivatives thereof having functionally equivalent properties, of the present invention may be used alone or in combination, and medicines comprising them as an active ingredient may be administered together with adjuvants or in particulate dosage form in order to effectively establish the cellular immunity. Specifically, when tumor antigen protein or tumor antigen peptide is administered to a subject, tumor antigen peptides are presented at high density on MHC class I antigens of the antigen-presenting cells, resulting in efficient proliferation of tumor-specific CTLs. For such purpose, those adjuvants described in the literature (*Clin. Microbiol. Rev.*, 7:277-289, 1994) are applicable. The active ingredient(s) are administered in a dosage form which allows the foreign antigen peptide to be efficiently presented on MHC class I antigen, such as liposomal preparations, particulate preparations in which the active ingredient(s) are bound to beads having a diameter of several  $\mu\text{m}$ , or preparations in which the active ingredient(s) are bound to lipids. It may be also possible to administer antigen-presenting cells such as dendritic cells or macrophages pulsed with the tumor antigen peptide, or cells transfected with DNA encoding the tumor antigen protein. Although the dose of the tumor antigen protein or tumor antigen peptide of the present invention in such preparations may be appropriately adjusted depending on various factors such as the disease to be treated, age and body weight of a particular patient, preferred dose is between 0.0001 mg and 1000 mg, and more preferably between 0.001 mg and 1000 mg. It is preferably administered every several days to



every several months.

A method for *in vitro* induction of CTL from peripheral lymphocytes using tumor antigen peptide of the present invention is exemplified as follows.

5           Peripheral blood lymphocytes from an esophageal cancer patient with squamous cell carcinoma are *in vitro*-cultured, and a tumor antigen peptide of the present invention, for example, a peptide having the sequence of "736-744", "749-757", "785-793", or "690-698" is added to the culture medium at the final concentration of 10 µg/ml, in  
10           order to stimulate the peripheral blood lymphocytes. Such stimulation is repeated three times at intervals of one week. One week after the third stimulation, the peripheral blood lymphocytes are recovered, and measured for their cytotoxic activity according to the methods described in D. D. Kharkevitch et al, Int. J. Cancer, 58:317 (1994), in order to  
15           find CTL-inducing activity of the tumor antigen peptide of the present invention.

          The method of the present invention for diagnosing tumors or autoimmune diseases may be conducted using antibodies specifically binding to a tumor antigen protein or tumor antigen peptide. Examples  
20           of such method may include those detecting tumor antigen protein in tumor tissue preparations, or detecting the presence of tumor antigen protein or antibodies against tumor antigen protein in blood or tissues. Such detection may be achieved by any appropriate method selected from, for example, immunohistochemical methods, immunoblotting,  
25           radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA),

fluorescent and luminescent assays. Furthermore, detection of tumor antigen protein using antibodies enables early detection of tumors or their recurrence, as well as selection of patients who may be suitably treated with the tumor antigen proteins, tumor antigen peptides, or DNA encoding them.

#### BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 is electrophoretograms showing the result of Northern blot hybridization described in Example 2.

In Fig. 1 a), KE-4, KE-3, TE-8, and TE-9 indicate esophageal cancer cell lines; Kuma-1 indicates a head and neck cancer cell line; HSC-4 indicates a mouth cancer cell line; Bec-1 indicates a B cell line; KMG-A indicates a gallbladder cancer cell line; R-27 indicates a breast cancer cell line; KIM-1, KYN-1, and HAK-3 indicate hepatic cancer cell lines; and M36 and M37 indicate melanoma cell lines.

#### EXAMPLES

The following detailed examples are presented by way of illustration of certain specific embodiments of the invention. The Examples are representative only and should not be construed as limiting in any respect.

##### Reference Example 1

Establishment of Cytotoxic T Lymphocyte (CTL) Cell Line against Esophageal Cancer Cell Line

According to the disclosure of Nakao *et al.*, *Cancer Res.*, 55:4248-4252 (1995), CTL against an esophageal cancer cell line, KE-4, belonging to squamous cell carcinomas when classified on the basis of

the tissue type was established from peripheral blood monocytes of a patient, named KE-4CTL, and used in experiments. The esophageal cancer cell line KE-4 and KE-4CTL have been deposited at The National Institute of Bioscience and Human Technology (1-1-3 Higashi, Tsukuba, Ibaraki, Japan) under International Deposition Nos. FERM BP-5955 and FERM BP-5954, respectively, both on May 23, 1997. Furthermore, typing of HLA class I molecules of KE-4 was conducted according to the above-noted disclosure of Nakao *et al.*, and it was confirmed that they are HLA-A2402, -A2601, B54, -B60, -Cw1, and -Cw3.

#### 10 Reference Example 2

##### Preparation of HLA-A2061 cDNA and HLA-A2402 cDNA

Using KE-4, a recombinant plasmid was prepared by incorporating cDNA for HLA-A2601 into an expression vector pCR3 (INVITROGEN) according to the disclosure of Nakao *et al.*, *Cancer Res.*, 55:4248-4252 (1995). Another recombinant plasmid for HLA-A2402 was also prepared in the similar manner.

#### Reference Example 3

##### Preparation of cDNA Library derived from KE-4

Poly (A)<sup>+</sup> mRNA was prepared from KE-4 by isolation of total RNA fraction and purification on oligo (dT) column using mRNA Purification system (manufactured by Pharmacia Biotech) according to the manufacturer's protocol. cDNAs having *Not* I adapter and *Sca* I adapter linked to each terminus were prepared from mRNAs using SuperScript<sup>TM</sup> Plasmid System (Gibco BRL) according to the manufacturer's protocol, and then ligated to an expression vector,

plasmid pSV-SPORT1 (Gibco BRL), digested with restriction enzymes *Not* I and *Sal* I, to yield recombinant plasmids. The recombinant plasmids were introduced into *E. coli*. ElectroMAX DH10B/p3<sup>TM</sup> cells (Gibco BRL) using electric pulses in Gene Pulser (Bio-Rad) under conditions of 25  $\mu$ F and 2.5 kV. Transformants into which the recombinant plasmids had been introduced were selected in LB medium (1% bacto-trypton, 0.5% yeast extract, 0.5% NaCl, pH7.3) containing ampicillin (50  $\mu$ g/ml).

#### Reference Example 4

##### Quantitative Determination of Interferon- $\gamma$

Quantitative Determination of interferon- $\gamma$  (IFN- $\gamma$ ) was conducted by enzyme immunoassay (ELISA). Anti-human IFN- $\gamma$  mouse monoclonal antibody as a solid-phased antibody was adsorbed on wells of 96-well microplate, and after blocking non-specific bindings with bovine serum albumin, allowed to bind with IFN- $\gamma$  in samples. Anti-human IFN- $\gamma$  rabbit polyclonal antibody as a detection antibody was then allowed to bind, and after binding with an anti-rabbit immunoglobulin goat antibody labeled with alkaline phosphatase, reacted with para-nitrophenyl phosphate as a chromogenic substrate. After stopping the reaction by adding an equal volume of 1N NaOH, absorbance at 405 nm was measured. The absorbance was compared with that obtained with standard IFN- $\gamma$  to determine the amount of IFN- $\gamma$  in the sample.

#### Example 1

##### Screening of Gene for Novel Tumor Antigen Protein

The recombinant plasmid DNAs were recovered from pools of about 100 transformants described in Reference Example 3 as follows.

A hundred transformants were introduced and cultured in each well of 96-well U-bottomed microplate containing LB medium plus ampicillin

5 (50 µg/ml). Part of the culture was then transferred to another 96-well U-bottomed microplate containing 0.25 ml per well of TYGPN medium (F.M. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.), and cultured for 48 hours at 37°C. The remaining cultures in LB medium on the microplate were stored in frozen.

10 Preparation of recombinant plasmid DNAs from transformants cultured in TYGPN medium was achieved in the microplate by alkaline lysis (F.M. Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.). The recombinant plasmid DNAs recovered by isopropanol precipitation were suspended in 50 µl of 10 mM Tris, 1 mM EDTA, pH  
15 7.4, containing 20 ng/ml RNase.

Fibroblast cell line, VA-13 cells (RIKEN CELL BANK, The Institute of Physical and Chemical Research; *Ann. Med. Exp. Biol. Fenn.*, 44:242-254, 1966) were doubly transfected with the recombinant plasmid for KE-4 cDNA and the recombinant plasmid for HLA-A2601  
20 cDNA using Lipofectin method as follows. Seven thousands VA-13 cells were placed in each well of 96-well flat-bottomed microplate, and incubated for 2 days in 100 µl of RPMI 1640 medium containing 10% FCS. Using Lipofectin reagent (Gibco BRL), 30µl of 70 µl mixture consisting of 25 µl of the recombinant plasmid for KE-4 cDNA

corresponding to about 100 transformants, 10  $\mu$ l (200 ng) of the recombinant plasmid for HLA-A2601 cDNA described in Reference Example 2, and 35  $\mu$ l of about 35-fold diluted Lipofectin reagent was added to VA-13 cells to be doubly transfected. Transfectants were prepared in duplicate. After 5 hours, 200  $\mu$ l of culture medium containing 10% FCS was added to the transfectants, and further incubated for 72 hours at 37°C. After removing the culture medium, 10,000 KE-4CTL cells were added to each well, and cultured for 24 hours at 37°C in 100  $\mu$ l of culture medium containing 10% FCS and 25 U/ml IL-2. The culture medium was recovered, and measured for IFN- $\gamma$  by ELISA.

Regarding four groups in which high production of IFN- $\gamma$  was observed, corresponding frozen-stored pools of about 100 transformants containing recombinant plasmids for KE-4 cDNA were used in the following screening. The pools of the transformants were plated on LB agar medium containing ampicillin (50  $\mu$ g/ml) to obtain colonies. Two hundreds colonies for each group (total 800 colonies) were cultured as described above so that a single kind of transformant is included in each well, thereby recombinant plasmid DNAs for KE-4 cDNA were prepared. Then, VA-13 cells were doubly transfected with the recombinant plasmid for KE-4 cDNA and the recombinant plasmid for HLA-A2601 cDNA followed by cocultivation with KE-4CTL, and IFN- $\gamma$  produced due to KE-4CTL reaction was quantitatively determined as described above in order to select positive plasmids. In this manner, a single KE-4

cDNA recombinant plasmid clone was selected and named 6DI.

Furthermore, similar procedures were repeated with 6DI to determine the amount of IFN- $\gamma$  produced by KE-4CTL according to the method described in Reference Example 4. The results are shown in the

5 following TABLE 1.

TABLE 1

Target cell	Amount of IFN- $\gamma$ produced by KE-4CTL (pg/ml)
VA-13 cell	0
VA-13 cell + HLA-A2601	1.8
VA-13 cell + 6DI	4.3
VA-13 cell + HLA-A2601 + 6DI	24.0
VA-13 cell + HLA-A0201 <sup>1)</sup>	0.9
VA-13 cell + HLA-A0201 + 6DI <sup>1)</sup>	3.0

<sup>1)</sup> For comparison, HLA of different type was transfected.

(These data was obtained by transfection using the following amounts

10 of DNA: 200 ng of HLA-A2601 or HLA-A0201, 100 ng of 6DI.)

### Example 2

#### Expression Analysis for Tumor Antigen Protein Gene by Northern Hybridization

15 RNAs were prepared from various cell lines using RNazol B (TEL-TEST, Inc.). Five  $\mu$ g of RNA was denatured in the presence of formamide and formaldehyde, electrophoresed on agarose, then transferred and fixed onto Hybond-N+ Nylon membrane (Amersham). As RNAs from normal tissues, commercially available membranes (Clontech) onto which mRNAs have been preblotted were used. The

inserted sequence region of the recombinant plasmid 6DI cloned in Example 1 was labeled with  $^{32}\text{P}$  using Multiprime DNA labelling system (Amersham) to prepare DNA probe. According to the known method (Nakayama *et al.*, *Bio-Jikken-Illustrated*, vol. 2, "Idenshi-Kaiseki-No-Kiso (A Basis for Gene Analysis)", pp. 148-151, Shujunsha, 1995), this probe was hybridized to RNAs on the membranes, and subjected to autoradiography to detect mRNA for tumor antigen protein gene of the present invention. The membranes used for the detection of mRNA for said gene were boiled in 0.5% aqueous sodium dodecyl sulfate to remove the probe, and subjected to Northern hybridization in a similar manner using  $\beta$ -actin as a probe which is constitutively expressed in cells, in order to detect mRNA which was used as an internal standard. The results are shown in Fig. 1. It became apparent from these results that mRNA for tumor antigen protein gene of the present invention is widely expressed in various cancer cells and normal tissues, and is about 2.5 kb in full length (Fig. 1).

### Example 3

#### Cloning and Base Sequencing of Full-Length cDNA Clone Encoding Tumor Antigen Protein

KE-4-derived cDNA Library described in Reference Example 3 was plated on LB agar medium containing ampicillin (50 $\mu\text{g/ml}$ ). The colonies thus obtained were then transferred and fixed on Hybond-N+ nylon membrane (Amersham) according to the manufacturer's protocol. The same 6DI probe as that used in Example 2 was employed for hybridization and autoradiography under the same conditions as those



used in Example 2, in order to select colonies which contain recombinant plasmids having the cDNA for tumor antigen protein gene incorporated. Furthermore, recombinant plasmids were recovered from the colonies selected, treated with restriction enzymes *Not* I and *Sal* I, and then electrophoresed on agarose to determine the length of incorporated cDNAs. A recombinant plasmid incorporating cDNA of about 2.5 kb was selected, and named K3. The base sequence of the cDNA region in this plasmid K3 was determined using DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer). The base sequence thus determined is shown in SEQ ID NO: <sup>1</sup>~~2~~. The full-length of the cDNA was 2527 base pairs. The amino acid sequence (800 amino acids) encoded by the base sequence of SEQ ID NO: <sup>1</sup>~~2~~ is shown in SEQ ID NO: <sup>2</sup>~~1~~.

The analysis indicated that the base sequence shown in SEQ ID NO: <sup>1</sup>~~2~~ does not show homology with known tumor antigen protein genes derived from melanomas and thus proved to be a different gene. The search for the base sequence of SEQ ID NO: <sup>1</sup>~~2~~ using WWW Entrez database revealed that part of the base sequence of the present invention exhibits high homology more than 90% to three gene sequences, functions of which are not known, decoded by WashU-Merck EST Project and registered at GENBANK under Accession Nos. R89163, R62890, and R00027. No. R89163 corresponds to the sequence of positions 1893-2267; R62890 corresponds to the sequence of positions 2018-2389; and R00027 corresponds to the sequence of positions 2024-2510. These sequences correspond, however, to the base sequences 3' to the initiation codon in the base sequence of the present invention, and

therefore, the amino acid sequences they encode can not be determined.

After determination of the base sequence as described above, the plasmid K3 was introduced into *E. coli* JM109 to obtain *E. coli* JM109(K3) which is a transformant for storage containing the novel tumor antigen protein cDNA of the present invention. *E. coli* JM109(K3) has been deposited at The National Institute of Bioscience and Human Technology (1-1-3 Higashi, Tsukuba, Ibaraki, Japan) under International Deposition No. FERM BP-5951 on May 22, 1997.

Furthermore, cDNA library derived from normal human tissue (peripheral blood lymphocyte) was also screened in the manner as described above. This screening resulted in cloning of a recombinant plasmid into which cDNA of about 2.5 kb has been incorporated. It was found by determining the base sequence of this cDNA that cDNA thus cloned was the same as that shown in SEQ ID NO: <sup>1</sup>/<sub>2</sub> except for position 812 (position 812 for normal human tissue was T). It was thus indicated that in connection with the full-length gene comprising the gene encoding the tumor antigen protein of the present invention, almost the same genes are expressed in both cancer cells and normal human tissue.

VA-13 Cells were then doubly transfected with the recombinant plasmid K3 incorporating cDNA for the novel tumor antigen protein gene and another recombinant plasmid incorporating cDNA for HLA-A2601, and used as target cells. The amount of IFN- $\gamma$  produced by the reaction of KE-4CTL was determined according to the method described in Reference Example 4. The results are shown in the

following TABLE 2.

TABLE 2

Target cell	Amount of IFN- $\gamma$ produced by KE-4CTL <sup>1)</sup> (pg/ml)
VA-13 cell + HLA-A2601 + K3	1439
VA-13 cell + HLA-A0201 <sup>2)</sup> + K3	10

5    <sup>1)</sup> Values obtained by subtracting the amount (background) of IFN- $\gamma$  produced by KE-4CTL in response to VA-13 cells transfected with each HLA.

<sup>2)</sup> For comparison, HLA of different type was transfected.

(These data was obtained by transfection of the following amounts of  
10 DNA: 200 ng of HLA-A2601 or HLA-A0201, 100 ng of K3.)

#### Example 4

##### Identification of Tumor Antigen Peptide

From the recombinant plasmid 6DI cloned in Example 1 which incorporated partial cDNA for the novel tumor antigen protein gene,  
15 plasmids containing partial cDNA for tumor antigen protein gene of various length were prepared using Deletion Kit for Kilo-Sequence (Takara Shuzo Co.) according to the manufacturer's protocol. These plasmids were introduced into *E. coli* ElectroMax DH10B/p3<sup>TM</sup> cells (Gibco BRL). The cells were plated on agar medium, and 50 colonies  
20 were selected at random. From the colonies, plasmid DNAs were prepared, subjected to electrophoresis, and 5 clones which contained plasmids having appropriate length selected.

According to the method described in Example 1, VA-13 cells were doubly transfected with HLA-A2601 cDNA and the above plasmid DNA, cocultured with KE-4CTL, and IFN- $\gamma$  in the culture medium was quantitatively determined according to the method described in

5 Reference Example 4. As a result, it was found that the transfectant with a plasmid lacking the base sequence after position 2253 in SEQ ID

<sup>1</sup>  
B No: ~~2~~ had no IFN- $\gamma$ -inducing activity on KE-4CTL. It was therefore suggested that peptides having the sequence after about position 739 in

<sup>2</sup>  
B the amino acid sequence of SEQ ID NO: ~~1~~ may have IFN- $\gamma$ -inducing  
10 activity on KE-4CTL.

Thus, a series of 21 different peptides each consisting of successive 10 amino acid residues in the amino acid sequence after position 730 in SEQ ID NO: <sup>2</sup>~~1~~ were synthesized so that they each have the amino acid sequence shifted serially by three amino acid residues.

15 Using these peptides, IFN- $\gamma$  in culture medium was determined as described above except that the antigen presentation was achieved by pulsing HLA-A2601 cDNA-transfected VA-13 cells with the peptides. As the result, IFN- $\gamma$ -inducing activity was observed in the peptides having the amino acid sequences of "736-745", "748-757", and "784-  
B 20 793" in SEQ ID NO: <sup>2</sup>~~1~~.

For each of these three peptides, additional peptides consisting of 9 amino acid residues were synthesized by truncating the N- or C-terminal residue, and used for measurement of IFN- $\gamma$ -inducing activity in a similar manner. Stronger IFN- $\gamma$ -inducing activity was

observed for the peptides having the amino acid sequences of "736-744",  
 "749-757", and "785-793" in SEQ ID NO: <sup>2</sup>~~1~~. The results are shown in  
 TABLE 3.

TABLE 3

Pulsed cell	Peptide	Amount of INF- $\gamma$ produced by KE4-CTL cells (pg/ml)
VA-13/A2601 <sup>1)</sup>	"736-744"	203
VA-13/A0201 <sup>2)</sup>	"736-744"	44
VA-13/A2601	"749-757"	183
VA-13/A0201	"749-757"	89
VA-13/A2601	"785-793"	394
VA-13/A0201	"785-793"	102

<sup>1)</sup> VA-13 cells transfected with HLA-A2601 cDNA

<sup>2)</sup> VA-13 cells transfected with different HLA-A0201 cDNA as a control

The results in TABLE 3 suggest that these peptides function as  
 a tumor antigen peptides.

In addition, it is known that there are certain rules (motifs) in  
 the sequences of antigen peptides bound and presented by HLA  
 molecules. Concerning the motif for HLA-A24, the second amino acid  
 is tyrosine and the ninth amino acid is isoleucine, leucine or  
 phenylalanine in the sequence of antigen peptides consisting of 9 amino  
 acid residues (*Immunogenetics*, 41:178-228, 1995).

Thus, another peptide having the amino acid sequence of  
 "690-698" in SEQ ID NO: <sup>2</sup>~~1~~ which corresponds to the above motif was  
 further synthesized. VA-13 cells transfected with HLA-A2402 cDNA

was then pulsed with the peptide, and IFN- $\gamma$ -inducing activity on KE-4CTL was measured as described above. The results are shown in TABLE 4

Pulsed cell	Peptide	Amount of INF- $\gamma$ produced by KE4-CTL cells (pg/ml)
VA-13	"690-698"	157
VA-13/A2402 <sup>1)</sup>	"690-698"	269
VA-13/A0201 <sup>2)</sup>	"690-698"	166

5 <sup>1)</sup> VA-13 cells transfected with HLA-A2402 cDNA

<sup>2)</sup> VA-13 cells transfected with different HLA-A0201 cDNA as a control

The results in TABLE 4 suggest that the peptide "690-698" functions as a tumor antigen peptide.

#### Example 5

10 Inducement of CTL from peripheral blood lymphocytes by tumor antigen peptides

The inventors have investigated whether antigen-specific CTL can be induced from peripheral blood lymphocytes of the cancer patient from whom KE-4 was derived, by *in vitro* stimulation with the tumor antigen peptides described in Example 3. Tumor antigen peptides used were those peptides having the sequences of "736-744", "749-757", and "690-698", obtained in the above Example 3. Frozen peripheral blood lymphocytes, which had been separated from the above cancer patient using Ficoll method, were awoke, transferred to 24-well plate at about 15  $2 \times 10^6$  cells/well, and cultured in RPMI 1640 medium containing 10% FCS and IL-2 (100 U/ml). To stimulate the peripheral blood

lymphocytes, the above tumor antigen peptide was added to the culture medium at 10 µg/ml. After one week, 10 µg/ml of the above tumor antigen peptide was added together with about  $1 \times 10^5$  cells of X ray-radiated (50 Gy) peripheral blood lymphocytes for the second stimulation. After additional one week, the third stimulation was conducted in a similar manner.

For peptides having the sequences of "736-744" and "749-757", peripheral blood lymphocytes were recovered one week after the third stimulation, and measured for their cytotoxic activity using, as target cells,  $^{51}\text{Cr}$ -labeled KE-4 and another esophageal cancer cell line KE-3 of which HLA-A loci are A2402 and A2, according to the method described in D.D. Kharkevitch *et al.*, *Int. J. Cancer*, **58**:317 (1994). The results are shown in TABLE 5.

TABLE 5

Effector cell	Target cell	Toxic activity (%)
Peripheral blood lymphocytes stimulated with "736-744"	KE-4	22.1
	KE-3	3.7
Peripheral blood lymphocytes stimulated with "749-757"	KE-4	35.9
	KE-3	24.2

When stimulated with the peptide having the sequence of "736-744", KE-4 was severely injured, whereas the negative control KE-3 was not injured. It was therefore demonstrated that CTL specific for KE-4 was induced. Similarly, when stimulated with the peptide having the sequence of "749-757", stronger cytotoxic activity was observed on KE-4, although nonspecific cytotoxic activity was also

observed on KE-3, suggesting that CTL specific for KE-4 was induced.

For peptide having the sequence of "690-698", peripheral blood lymphocytes were recovered after the third stimulation, and further cultured in RPMI-1640 medium containing 10% FCS, 50% AIM-V (Gibco BRL), and IL-2 (100 U/ml). Then, the cytotoxic activity was measured as above using  $^{51}\text{Cr}$ -labeled KE-4 and VA-13 cells as target cells. In addition, lymphocytes were isolated from peripheral blood of a normal individual of which HLA-A loci were homozygous A24, and measured for their cytotoxic activity in the same manner as above using, as target cells,  $^{51}\text{Cr}$ -labeled KE-4 and lung cancer cell line QG-56 of which HLA-A loci are homozygous A2601. The results are shown in TABLE 6.

TABLE 6

Effector cell	Target cell	Toxic activity (%)
"690-698"-Stimulated peripheral blood lymphocytes from a cancer patient	KE-4	24.7
	VA-13	13.8
"690-698"-Stimulated peripheral blood lymphocytes from a normal individual	KE-4	17.7
	QG-56	11.5

By stimulating peripheral blood lymphocytes from a cancer patient and from a normal individual with the peptide having the sequence of "690-698", stronger cytotoxic activity was observed on KE-4, although nonspecific cytotoxic activity was also observed on the negative controls VA-13 and QG-56 cells. The above results suggest that CTLs specific for KE-4 were induced.



**EFFECTS OF THE INVENTION**

According to the present invention, there are provided medicines for activating antitumor immunity by means of tumor antigen proteins and tumor antigen peptides, medicines for treating autoimmune diseases, and medicines comprising DNA or the like encoding tumor antigen protein, as well as methods for diagnosing tumors or autoimmune diseases.

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